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ARTICLE TYPE

# Supramolecular Three-Component Amino Acid-Based Hydrogels with Superior Mechanical Strength for Controllable Promoting Nonpathogenic *E. Coli* Growth

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Novel three-component hybrid hydrogels have been constructed by amino acid derivatives, riboflavin and melamine through self-assembly. In the process of gelating, the drive forces are attributed to be hydrogen bonding and π-π interaction. These luminescent hydrogels demonstrate excellent mechanical strength (>$10^4$ Pa) and low cell toxicity, which have been investigated by means of ultraviolet spectra (UV), fluorescence spectra, scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM), rheological experiments and cytotoxicity assay. What's more, these hybrid hydrogels could be used to drive the drug release in a controlled manner and be used for promoting *E. coli* growth, which accelerate the growth of *E. coli* distinctly.

## 1. Introduction

Supramolecular hydrogels *via* various non-covalent interactions have great applications in mechano-responsive sensor materials[1], drug delivery vehicles, [2, 3] biomedicine, [4] cell culture[5] and shape memory materials.[6] In comparison with the conventional polymer hydrogels, supramolecular hydrogels are more apt to be regulated for their thermo-reversibility, stimuli-responsiveness, and biocompatibility since the hydrogelators can be derived from biological components including amino acids, sugars and steroids. Such materials are responsive to variety of stimulus such as pH, [7-9] light, [10] vigorous agitating,[11] temperature [12, 13] and enzymes.[14, 15] Recently, a great deal of multi-component hydrogels have been reported.[16-18] This class of hydrogels is a means of creating responsive materials with enhanced functionalities. It remains a challenge to construct supramolecular hydrogels with superior mechanical strength and excellent biocompatibility.

As an important driving force, complementary hydrogen bonding is a relatively strong non-covalent interaction in multicomponent hydrogel systems during the self-sorting process. Especially, molecules such as melamine and cyanuric acid (CA) or their derivatives can form a stable complex which displays three complementary NH–O and NH–N hydrogen bonds through the interaction between diaminopyridine and diimide moieties.[19-23] Such triple hydrogen bonding is responsible for the enhanced strength and stability of gels due to its specificity and directionality. Riboflavin with the conjugated aromatic moieties named flavin nucleotides behaves as a photoreceptor in the phototropism of plants [24], plays a significant role in various biochemical processes in plants, [25] and also acts as a perfect component in hydrogel systems.[26-28] Part of such hydrogel systems are suitable for biomedicine and for designing new fluorescence sensors.[29, 30]

In addition, hydrogelators consist of aromatic moieties may be beneficial to improve strength rely on aromatic-aromatic interactions.[31-35]As a charming class of chromophores and fluorophore, perylenetetracarboxylic diimides (PDI) are excellent building blocks for self-organized molecular materials on account of the strong π–π stacking between the planar PDI cores.[36, 37]

What's more, amino acids or peptides with low biotoxicity and good biocompatibility have been recognized as very useful building blocks for the construction of biomaterials. Amino acid or peptide-based functionalized building blocks for generating hydrogels have been reported recent years.[38-41] Herein we utilize amino acid derivatives, riboflavin and melamine to construct luminescent hydrogels *via* self-assembled behavior and then to explore mechanical properties, drug release process and the capability of promoting nonpathogenic *E. coli* growth.

## 2. Experimental section

### 2.1 Materials

All starting materials were purchased from commercial suppliers and used without any further processing. Double distilled water (DDW) was used in all experiments.

### 2.2 Characterization

NMR spectra were measured on a Bruker ULTRASHIELD 400 ($^1$H NMR 400 MHz) spectrometer. UV absorption spectra were obtained using a UV-2700 UV-Vis spectrophotometer. The luminescence spectra were measured on a LS55 fluorescence spectrophotometer. The path length of the quartz cell is 1 cm, while the emission bandwidth was 5 nm. The FT-IR spectra were recorded on an AVATAR 360 FT-IR spectrophotometer. The powdered samples were mixed with KBr to prepare the thin films in the solid-state FT-IR studies. SEM images of these samples were recorded using FEI QUANTA 450 with accelerating voltage 15.0 kV. Samples were obtained through dropping the gels on a flat surface of a cylindrical aluminum substrate and allowed to dry at room temperature. Then the samples were coated with gold using a MSP-1S magnetron sputter (Japan) coater. Rheological characterization was performed using a stress controlled rheometer (HAAKE RheoStress 6000) with parallel plate type

geometry (plate diameter, 3.5 cm). A solvent trap equipped with rheometer was used to protect the sample from evaporation. Frequency sweeps at 25 □ were carried out over a range of 0.1-100 rad $s^{-1}$ at strain amplitude of 1%. Confocal laser scanning microscope (CLSM) images were taken with confocal laser scanning microscope FV 1000 invert microscope (OLYMPUS) under 100X magnification at an excitation wavelength of 488 nm.

**2.3 Gelation**

The gelation tests of amino acid functionalized perylene derivatives with riboflavin and melamine were carried out, respectively. Gelation was determined by the absence of flow when the tube was inverted at room temperature, respectively. To prepare the hydrogel system, a weighed sample of GP, riboflavin and melamine at the molar ratio of 1/2/2, 1/2/0.1, 1/2/3, 1/1/0.1, 1/2/0.05were put into a septum-capped vial (1.5 mL) with 1.0 mL $H_2O$. Then the samples were executed with ultrasonic-processing continuously at room temperature. After gelating, the samples were allowed to take out and the gelating time was recorded with the ultrasound turning off. The gelation of TP/RF/MM is similar to the GP/RF/MM system.

**2.4 Cell culture and cytotoxicity assay**

HeLa cells were seeded in 96-well plates at a density of $1 \times 10^5$ cells per well and then incubated overnight in DMEM containing 10% fetal bovine serum (FBS). The cells were washed with PBS and then incubated in fresh medium containing GP/RF/MM (10, 20, 50, 100 μM) for 24 h. The media were washed with PBS then the cells were incubated in DMEM containing tetrazolium dye (MTT) (0.25 mg $mL^{-1}$) and cultured for 4 h at 37 °C. After removal of the supernatant, DMSO (100 μL) was added to each well to dissolve the formazan crystals. The plates were shaken for 5 min and then analyzed with a SpectraMax M5 plate reader to record the absorbance at 490 nm.

**2.5 In vitro drug release**

RF released into the fluids was determined from the measurement of absorbance at 444 nm and a standard curve and expressed as follows:

RF release (%) = released RF/total RF×100 %

The released RF was calculated from the RF concentration (μg/mL) measured in the total solution volume and total RF was the amount gelated in hydrogel. All experiments were done in triplicate in amber bottles to prevent photodecomposition of RF.

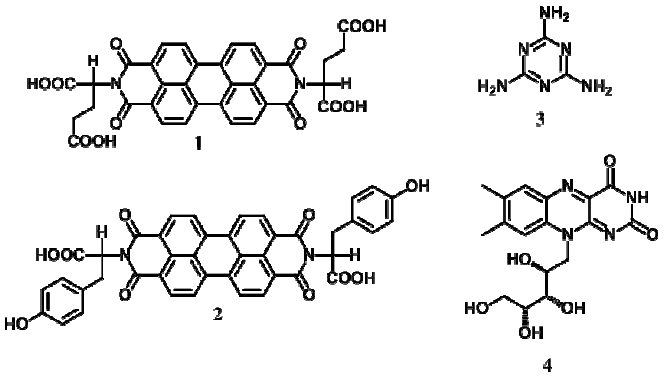
The total RF is 7.52 mg, and the amber bottles were total filled with 100 ml PBS (0.1M, pH 7.4) at 37 ℃. During the in vitro release experiment, 0.5 ml the buffer medium was removed from the amber bottles and the concentration of RF was measured by means of a UV spectrophotometer (UV-2700 UV-Vis spectrophotometer) at 444 nm. A 0.5 ml fresh buffer solution was added back to the amber bottles to maintain the same total solution volume.

**2.6 Bacteria Culture**

Bacteria of *Escherichia coli* (DH5α) were utilized in bacteria culture experiments. Prior to the experiments, the bacteria were grown overnight in Luria–Bertani broth medium (LB). Then 5 μL of bacteria LB solution (logarithmic phase) was introduced into the solution of the supramolecular hydrogel at different concentration (0, 5, 10, 20, 50, 70, 100, 150 μg/mL). The 96-well tissue culture plates containing solution of gels and bacteria cultures were placed on a shaker at 37 □, and the OD readings of bacteria solutions were monitored every 2- 4 h by measuring the $OD_{630}$ with a Bio-RAD imark Microplate reader. The bacteria solution without hydrogel was used as control.

## 3. Results and discussion

The molecular structures of GP, TP, MM and RF were shown in scheme 1. The GP and TP had been synthesized and characterized in Scheme S1. The structures of GP and TP demonstrated that a perylene core and two amino acid residues were contained as terminal groups. These compounds belonged to a charming kind of chromophores and fluorophores due to the good optical properties and pronounced capabilities of π-π staking. The amino acid groups with admirable properties of being harmless and biocompatible might be contributed to the gelation process by hydrogen bonds. According to the method of gelating described in experimental section, we tested the gelation ability of compounds GP and TP with riboflavin (RF) and melamine (MM) in water, respectively.




**Scheme 1** Molecular structures of (1) glutamate -functionalized perylene derivatives (GP); (2) tyrosine -functionalized perylene derivatives (TP); (3) melamine (MM) and (4) riboflavin (RF).

It was found that GP/RF/MM system at the molar ratio of 1/2/2, 1/2/0.1, 1/1/0.1 and 1/2/3 were capable of generating stable, crimson and self-supporting hydrogels under ultrasound at room temperature, while GP/RF/MM (1/2/0.05) and TP/RF/MM systems (1/2/2 and 1/2/0.05) were unable to form gels under similar conditions (Fig. S1b). Meanwhile, any two components of GP/RF/MM system failed to generate gel under ultrasound at room temperature (Fig. S1a). What's more, as the control, the GP/RF/MM (1/2/2) system couldn't generate gel without ultrasound at room temperature (Fig. S1b). It was clear that ultrasound was necessary for gelation, which had an influence on the aggregation properties of GP/RF/MM in the solvents at room temperature. It was speculated that ultrasound provided sufficient energy to reorient the intermolecular hydrogen bonding, to extend π-π stacking interactions and then to elongate the fibers to form network structures. The gels could maintain gelating for about 2 month in the dark, which deduced that the perfect stability was closely related to network structures. To probe the proper molar ratio of the GP/RF/MM system, rheology experiments were carried out. The results exhibited that the appropriate ratio of GP/RF/MM was 1/2/2 (0.36% w/v) due to its higher intensity (Fig. S2c).Thus, GP/RF/MM gel system (1/2/2) was appropriate to explore the properties.

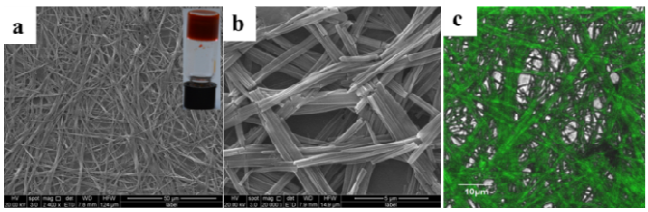



**Fig. 1** SEM images of the xerogels: (a) for GP/RF/MM hydrogel ([GP] $=10^{-2}$ M) in water in larger scale [insert image was photos of the gel]; (b) for GP/RF/MM hydrogel ([GP] $=10^{-2}$ M) in water in small scale; (c) CLSM images of GP/RF/MM hydrogel (overlay image).

Scanning electron microscopy (SEM) had been used to observe the structures of the gel. The images in Fig. 1 showed that the dried hydrogel was composed of fibrous structures. The GP/RF/MM xerogel generated a close-knit morphology, in which thin and small fibers with the diameter ranging from 200 to 700 nm cross-linked with each other to form three-dimensional networks (Fig. 1a and 1b). Additional, the porous between fiber bundles were small enough to keep solvents from flowing out, which led to the stability of gel. The enhanced fluorescence of hydrogel could be observed under a confocal laser scanning microscope (CLSM) upon excitation at 488 nm. Interestingly, highly dense entangled network structures comprised long and bundled fibers with bright fluorescent signal were observed in Fig. 1c. The morphology of these structures was consistent with the SEM images, which suggested that the hydrogel were luminescent gel with strong fluorescence (Fig. S2a and 2b).

To explore the driving force of the gel system, UV-Vis spectra, fluorescence spectra and infrared spectra were recorded. UV-Vis absorption spectra of GP/RF/MM=1: 2: 2 in $H_2O$ at concentration of [GP] = $1.0\times10^{-5}$ M were obtained by means of UV-Vis spectrophotometer. The spectrum of pure GP exhibited three intense absorption maxims at about 498 nm and 534 nm, while RF showed absorption peaks at 373 nm and 445 nm. The 373 nm peak was for the mixing of n-π* transition coupled with π–π* transition of riboflavin and the 445 nm peak was ascribed to π–π* transition.[42] However, the absorption spectrum of GP/RF/MM=1: 2: 2 solution displayed slight difference, in which the absorption band of 445 nm manifested somewhat red-shift and the absorption band of 498 nm disappeared (Figure 2a). It was due to the almost identical stacking geometry of the aromatic systems between RF and GP, and the aggregation of the GP/RF/MM system.

The fluorescence spectra of gel and single component solution had been investigated and showed in Fig. 2b. Compared to spectrum of GP ([GP] = $1.0\times10^{-5}$ M), the fluorescence intensity of GP/RF/MM=1: 2: 2 ([GP] = $1.0\times10^{-5}$ M) decreased sharply with emission peaks at 548 nm and 585 nm exhibiting no significant shift. According to the previous research of chemists, perylene diimide derivatives emitted enhanced fluorescence in dilute solution, while the fluorescence tended to become faintly or even quenching in aggregated or solid states.[43-45] The lower fluorescence intensity of the dilute gel could be ascribed to the formation of aggregation in water with the assistance of the π–π electronic coupling.

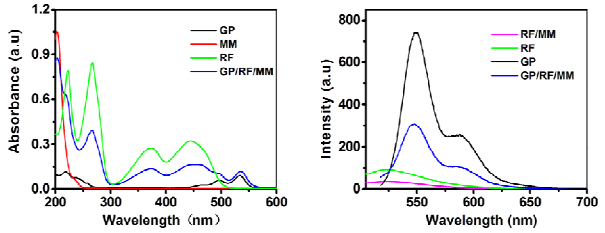



**Fig. 2** (a) UV-Vis spectra of GP/RF/MM = 1: 2: 2 system at room temperature. [PP] =$10^{-5}$ M and [RF] = [MM] = 2 .0× $10^{-5}$ M [path length = 2.0 mm]; (b) fluorescence spectra of solution and gel at room temperature ($\lambda_{ex}$ = 499 nm [path length = 5 mm]).

The infrared spectra could provide some important evidence for intermolecular interaction in the gelation, especially the contribution of hydrogen bonds provided by our compounds. In the figure, strong C=O band at 1751 $cm^{-1}$ and 1696 $cm^{-1}$ in pure GP were observed, which indicated that there was no strong hydrogen bond between molecules. However, a single band at 1691 $cm^{-1}$ was found in xerogel (Fig. S4), which was attributed to hydrogen bonded C=O vibration. The phenomenon illustrated the formation of the hydrogen bonding in the gel state.[46, 47] The above result indicated that the intermolecular interaction among riboflavin, melamine and GP played important roles during the gelation. From the spectra analysis mentioned above, it was evident that the gelator molecules were self-assembled through triple hydrogen-bonding interactions between RF and MM, hydrogen-bonding between RF and acid- functionalized perylene derivatives.

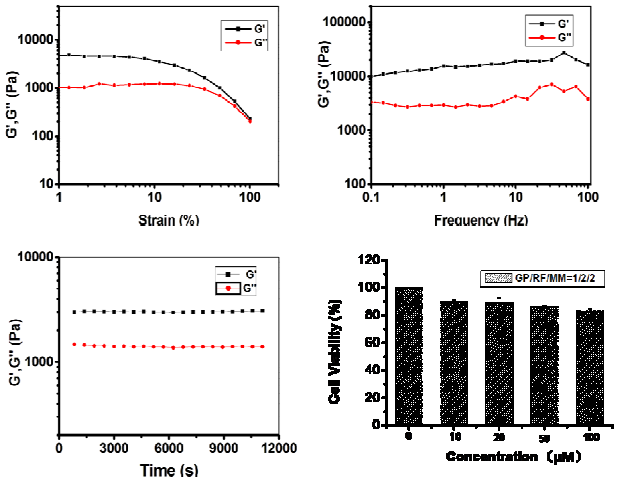



**Fig. 3** (a) strain sweep of the GP/RF/MM gel in $H_2O$ at a frequency of 6.28 rad $s^{-1}$; (b) frequency sweep of the GP/RF/MM gel at a strain of 0.1%; (c) time sweep of the GP/RF/MM gel at a strain of 0.1%; (d) 24 h cell viability test of GP/RF/MM (molar ratio of 1/2/2) at various ratios in different concentration.

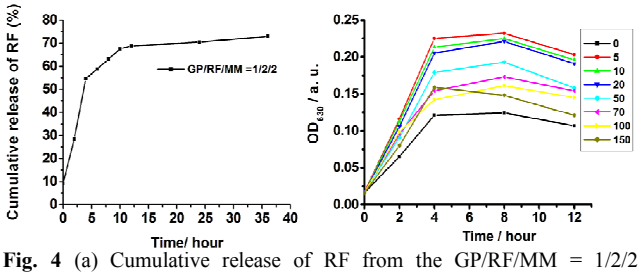



**Fig. 4** (a) Cumulative release of RF from the GP/RF/MM = 1/2/2 hydrogel at 37 ℃ in PBS solution (pH =7.4, 0.1 M); (b) Time-dependent the growth of *E. coli* in the solution of GP/RF/MM system (1/2/2) at different concentration (0, 5, 10, 20, 50, 70, 100, 150 μg/mL).

To discover the mechanical properties of the gel, rheology experiments were performed by rheometer. According to Fig. 3, the linear viscoelastic region (LVR) of the hydrogel was determined by strain amplitudes ranging from 0.01% to 100% at 6.28 rad $s^{-1}$. Strain sweep measurements illustrated the performance of hydrogels at different strains. Below a certain strain, the storage modulus G' and the loss modulus G'' were independent of the strain and the deformation was close to 0 with G' much higher than G'', which inferred that the gel structure kept completely intact. However, G' and G" dropped gradually and the gel was deformed to a certain extent (Fig. 3a) when beyond a certain strain. Frequency sweep exhibited typical solid-like rheological behavior with the storage modulus G' dominating the loss modulus G'' over the investigated oscillating frequency (Fig. 3b). Values of G' for GP/RF/MM gel were over $10^4$ Pa (Fig. 3b), which demonstrated that the hydrogel was remarkably strong, and potentially suitable for a wide range of different mechanical and medical applications. Rheological measurements of time sweep indicated that the viscoelasticity of hydrogels undergo

mainly minor changed over 12000 seconds at a frequency of 6.28 rad $s^{-1}$ and a strain of 30% (Fig. 3c). The robustness of the supramolecular gel was more than likely due to the enhanced hydrogen bonding and π–π interaction of the system, and due to the ultrasound time.

In order to test the biocompatibility of the gelator, MTT assay was used to evaluate the cytotoxicity of it on HeLa cells. A cytotoxicity diagram was obtained after 24 h incubation of HeLa cells with various amounts of hydrogel (0, 10, 20, 50, 100 μM) at different ratios. According to the results of the MTT assay shown in Fig. 3d, after being incubated with hydrogels whether at 10 μM, 20 μM, 50 μM or even 100 μM, the cell viability still remained above 85% (GP/RF/MM = 1/2/2). The value of $IC_{50}$ was still >100 μM despite the cell viability decreases slightly when they were incubated with GP/RF/MM = 1/2/0.1. These results illustrated that the hydrogelators at various ratios were of biocompatibility.

Fig. 4a showed the release of RF from GP/RF/MM hydrogels in PBS solution (pH = 7.4, $I$ = 0.1 M). It was observed that there was a burst release at the initial stage with the reason of immediately release of RF expose on the surface of the hydrogels as soon as the hydrogel was placed into the buffer solution, which have been reported in other literature.[48-50] Then the hydrogels were exhausted after the initial release of the RF expose on the surface, after that the consequent release seemed to be a diffusion process. After a period of time, the release slowed down with the release rate reaching at about 80% after 36 h in PBS solution for the reason that the driving force of RF diffusion named concentration gradient was gradually reducing.[51] The burst release was attributed to the higher RF concentration gradient between the hydrogel surface and the release medium during the early stage. It was inferred that the stability of hydrogel with the strong triple hydrogen bonding between RF and MM, hydrogen bonding and the π–π stacking interaction, which locked the RF from releasing to PBS solution. As a result, the release of RF trended to be slightly inefficient.

To test bacterial growth in the presence of biofunctional hydrogels, *Escherichia coli* (DH5 α) culture experiments were carried out. The *E. coli* (DH5 α) was incubated with different concentration of hydrogel (dissolved in LB solution) and the microbial proliferation was assessed by optical density (OD) measurement. Fig. 4b showed the time-dependent microbial proliferation test of the GP/RF/MM hydrogel. It was found that the OD values of all the experimental groups increased sharply in 2-4 h and almost reached the maximum, then grew moderately. As shown in the picture, the OD values reached the maximum after incubating for 8 hours and then slowly decreased. Interestingly, when incubating with various concentration of GP/RF/MM, the OD values generally greater than the control of which the bacteria solution incubated without hydrogel. As the concentration of hydrogel increasing, the promotions tended to decrease. From the figure, it was found that the optimal concentration was 5μg/mL, which deduced that the riboflavin (RF) was a kind of nutrients but not much-needed for *E. coli*.[52] In a word, the presence of the GP/RF/MM hydrogel accelerated bacterial growth completely. After incubating with *E. coli* for 32 h, the nanofiber structures of GP/RF/MM hydrogel disappeared (Fig. S3c), which demonstrated that *E. coli* had interacted with hydrogel.

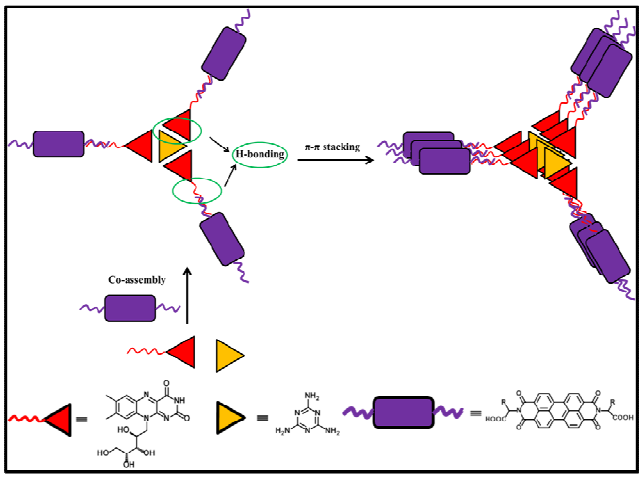



**Fig. 5** Proposed mechanism of self-assembling process.

Based on experimental results mentioned above, a model was proposed as shown in Fig. 5. RF and MM self-assembled through triple hydrogen bonding in water with the assistance of ultrasound. Meanwhile, four carboxylic groups of GP combine with RF *via* the hydrogen bonding interaction. Then assembled spontaneously based on π- π interaction to successfully from nanofibers to stable 3D networks. The self-assembly was the mainly due to the synergy effect of hydrogen bonding interaction and π- π interaction. When under low concentration, the melamine acted as a cross-linker, which led to the formation of hydrogels(GP/RF/MM = 1/1/0.33) (Fig. 5). When the concentration of melamine increasing, more cross-linking points were forming, which resulted in higher G' values of the gels (GP/RF/MM = 1/2/2) with the assembling process mentioned in Fig. S5. However, TP with two carboxylic groups had less opportunity to generate hydrogen bonds in the control experiment. This was due to the two p-hydroxyphenyl groups of TP hindered the π–π stacking during the hydrogelating process, which resulted in the failure to form networks.

## 4. Conclusions

We had discovered three-component luminescent hydrogels, which were composed of amino acids-functionalized perylene derivatives, riboflavin and melamine. The triple hydrogen bonding, hydrogen bonding and the π–π stacking from acid-functionalized perylene derivatives played vital role in the gel systems. The hydrogels exhibited excellent mechanical strength, biocompatibility and favorable sustained release effect. What's more, the presences of the hydrogels accelerated the growth of *E. coli* completely during the bacteria culture experiments. Such properties opened the possibility of the hydrogels to become potential materials in the field of biomedicine.

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## Notes and references

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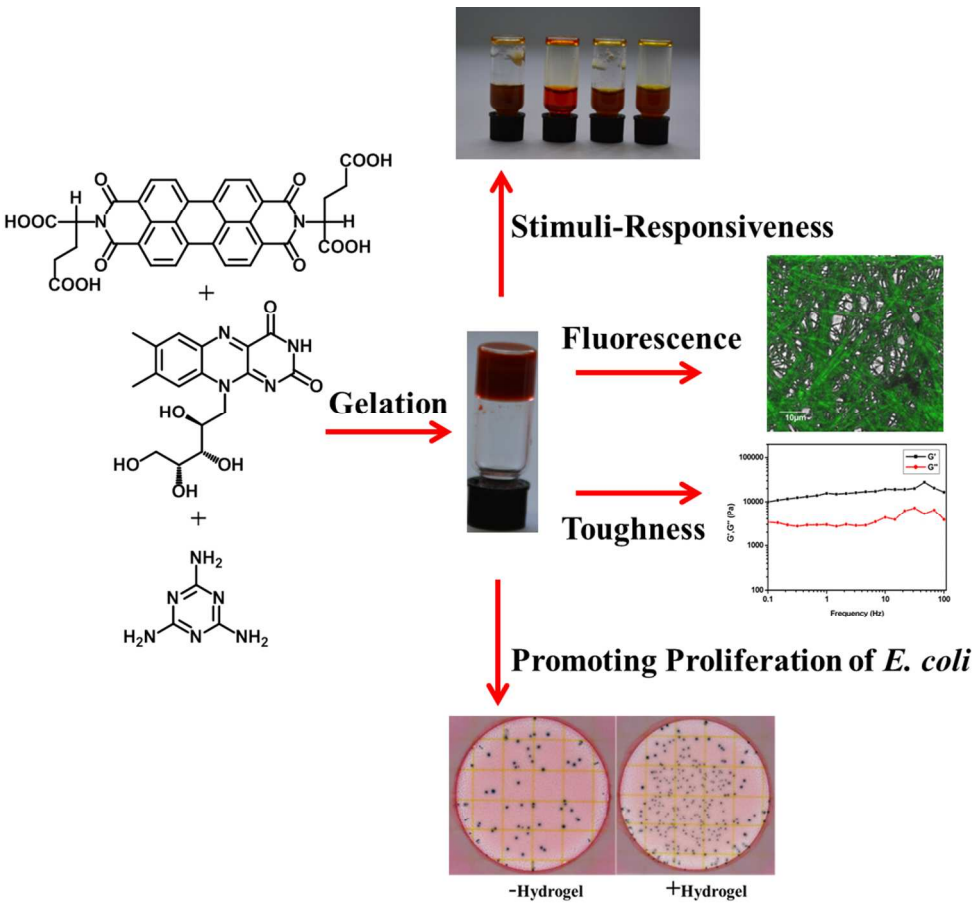
Novel three-component hybrid hydrogels have been constructed by amino acid derivatives, riboflavin and melamine through self-assembly, which demonstrate excellent mechanical strength (>104 Pa) and low cell toxicity.

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